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# Survivin as a therapeutic target in Sonic hedgehog-driven medulloblastoma

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## Abstract

Medulloblastoma (MB) is a highly malignant brain tumor that occurs primarily in children. Although surgery, radiation and high-dose chemotherapy have led to increased survival, many MB patients still die from their disease, and patients who survive suffer severe long-term side effects as a consequence of treatment. Thus, more effective and less toxic therapies for MB are critically important. Development of such therapies depends in part on identification of genes that are necessary for growth and survival of tumor cells. Survivin is an inhibitor of apoptosis protein (IAP) that regulates cell cycle progression and resistance to apoptosis, is frequently expressed in human MB, and when expressed at high levels predicts poor clinical outcome. Therefore, we hypothesized that Survivin may play a critical role in growth and survival of MB cells and that targeting it may enhance MB therapy. Here we show that Survivin is overexpressed in tumors from *patched* (*Ptch*) mutant mice, a model of Sonic hedgehog (SHH)-driven MB. Genetic deletion of *survivin* in *Ptch* mutant tumor cells significantly inhibits proliferation and causes cell cycle arrest. Treatment with small molecule antagonists of Survivin impairs proliferation and survival of both murine and human MB cells. Finally, Survivin antagonists impede growth of MB cells *in vivo*. These studies highlight the importance of Survivin in SHH-driven MB, and suggest that it may represent a novel therapeutic target in patients with this disease.

### Keywords

medulloblastoma; survivin; sonic hedgehog; targeted therapy; animal model; brain tumor

## Introduction

Medulloblastoma (MB) is the most common malignant brain tumor in children(1). Intensive therapy – including surgery, cranio-spinal radiation and high dose chemotherapy – has improved 5-year survival rates(2), but almost a third of MB patients still die from their disease, and survivors suffer severe long term side effects that affect their quality of life(3). Thus, safer and more effective therapies are needed for this disease.

In recent years, targeted therapies have begun to be evaluated in patients with MB. For example, with the recognition that a subset of MBs results from mutations in the Sonic Hedgehog (SHH) pathway(4), antagonists of Smoothened (SMO), an activator of the pathway, have advanced into clinical trials for the disease. Although initial reports suggested that these agents can inhibit tumor growth(5), as with many targeted therapies, resistance develops quickly(6). Moreover, patients who have SHH pathway mutations downstream of SMO do not respond to these agents at all(7). Thus, even for SHH-driven MBs, additional approaches are necessary.

Finding novel therapeutic targets for MB depends on identification of genes that are critical for tumor growth and survival. SURVIVIN (also known as baculoviral inhibitor of apoptosis

repeat-containing 5, or *BIRC5*), is a member of the IAP family that regulates both cell cycle progression and cell survival(8, 9). SURVIVIN is expressed in many human cancers, and its expression is correlated with poor clinical outcome in non-small cell lung cancer (NSCLC) (10), breast cancer(11), leukemia(12, 13), neuroblastoma(14) and glioma(15). Because of its high expression in tumors and minimal expression in most adult tissues(16), a number of approaches have been developed to inhibit its expression and function. These include small molecule antagonists, antisense oligonucleotides, and immunotherapy(17-21). Some of these approaches are in clinical trials for numerous cancers, including prostate cancer(22), melanoma(23, 24), breast cancer(25), and NSCLC(25, 26).

SURVIVIN has not been studied extensively in the context of MB. Although some studies have suggested that elevated expression is linked to poor prognosis(27-29), little is known about its role in MB growth and survival. Using an animal model of SHH-driven MB, we now show that Survivin is highly expressed in tumors and absent from normal adult cerebellum. Moreover, through both genetic deletion and pharmacological inhibition we demonstrate that Survivin is critical for proliferation and survival of mouse and human SHH driven MB cells. Finally, Survivin antagonists impair growth of MB in vivo, highlighting the potential of Survivin as a therapeutic target in patients with MB.

## Results

### Survivin is highly expressed in medulloblastomas from *Ptch* mutant mice

To determine whether Survivin could represent a target in SHH driven MB, we isolated RNA from *Ptch* mutant tumors and examined *survivin* expression using real time PCR. High levels of *survivin* were detected in all tumors and in granule neuron precursors (GNPs), the progenitors from which these tumors are thought to arise(30) (Figure 1A). Importantly, expression could not be detected in normal adult cerebellum. Similar results were seen when Survivin protein was examined by immunoblotting (Figure 1B). Staining of tissue sections revealed Survivin expression in the nuclei of tumor cells (abrogated by blocking peptide (Figure 1D)), and minimal staining in normal adult cerebellum (Figure 1C-F). These data indicate that Survivin is highly expressed in *Ptch* mutant tumors, raising the possibility that it might play an important role in tumor growth or maintenance.

### Survivin is critical for MB cell proliferation and cell cycle progression

To investigate the importance of Survivin for growth of MB cells, we first utilized a genetic approach. *Survivin*$^{fl/fl}$ mice(31), in which the *survivin* gene is flanked by loxP sites, were crossed with *Ptch*$^{+/-}$ mice, to generate tumors in which *survivin* can be deleted by Cre recombinase. We confirmed efficient deletion of *survivin* by isolating tumor cells from *Survivin*$^{fl/fl}$*;Ptch*$^{+/-}$ (SP) mice and infecting them with Cre retroviruses. After 48hrs, *survivin* expression was significantly reduced (by 82%) in Cre-infected cells compared to control (GFP-infected) cells (Figure 2A). We then looked at the effect of *survivin* loss on proliferation. After Cre-mediated deletion of *survivin* from SP tumor cells, thymidine incorporation was decreased by almost 90% (Figure 2B). Importantly, when tumor cells from *Survivin*$^{WT}$ mice were infected with Cre viruses, there was no appreciable difference in proliferation compared to control cells (Figure 2C), indicating that the decreased thymidine

incorporation observed in SP tumor cells was not due to non-specific toxicity of the Cre virus. To address whether loss of *survivin* affects cell cycle progression, we isolated cells from SP tumors, infected them with Cre or GFP viruses, and performed cell cycle analysis (Figure 2D,E). *survivin* deletion led to a marked accumulation of cells in the G2/M phases of the cell cycle (39% of Cre-infected cells vs. 9.5% of control cells in G2/M). Together, these data demonstrate that Survivin is necessary for proliferation and cell cycle progression of MB cells.

### Survivin antagonists inhibit MB cell proliferation and promote apoptosis

Given the importance of Survivin for MB proliferation, we hypothesized that pharmacological agents that inhibit Survivin expression or function might interfere with tumor growth. To test this, we obtained several small molecule Survivin antagonists: YM155 is an inhibitor of *survivin* transcription(32), whereas S12 and LLP3 bind directly to Survivin protein and interfere with its function(33, 34). To test the ability of YM155 to inhibit *survivin* expression in *Ptch* mutant MB cells, we treated cells with the drug for 48hrs, isolated RNA and performed qRT-PCR for *survivin*. YM155 markedly decreased *survivin* expression even at a concentration of 10 nM (Supp. Figure 1A). Similarly, loss of Survivin was detected at the protein level using western blotting (Supp Figure 1B). These data suggest that YM155 effectively inhibits *survivin* expression in *Ptch* mutant MB cells. To test the effects of Survivin antagonists on MB growth, we treated tumor cells with these agents and analyzed the percentage of cells expressing the proliferation marker Ki67. Consistent with our genetic results, inhibition of Survivin using either YM155 or S12 caused a significant decrease in the number of Ki67+ cells compared to treatment with vehicle (DMSO) (Figure 3A-D). Additionally, we saw a dose dependent decrease in thymidine incorporation after treatment with YM155, S12, or LLP3 (Figure 3E-F and Supp. Figure 2). These data suggest that Survivin antagonists effectively inhibit MB growth *in vitro*.

To determine whether Survivin antagonists also cause cell cycle arrest in MB cells, we performed cell cycle analysis. ~~A~~After 24hrs, cells treated with S12 showed a significant accumulation in G2/M (56%) compared to cells treated with vehicle (12%) (Figure 4A,C). G2/M accumulation was also seen at 36h (52%) (Figure 4B,D). In contrast to S12, YM155 decreased the percentage of cells in G2/M (7%), with a concomitant increase in S phase (from 15 to 20% at 24hr) (Figure 4E-H). Notwithstanding these differences, our data demonstrate that both Survivin antagonists alter normal cell cycle progression of MB cells.

In addition to its role in regulating cell cycle progression, Survivin has been suggested to function as an inhibitor of apoptosis. To determine if Survivin inhibition promotes MB cell apoptosis, tumor cells were treated with Survivin antagonists and then stained with Annexin-V and propidium iodide (PI). As shown in Figure 4E-G, antagonists increased the percentage of apoptotic (AnnexinV+) tumor cells from 21% (after DMSO treatment) to 62% (S12) or 59% (YM155). These data show that Survivin antagonists are not merely cytostatic, but can promote apoptosis of tumor cells as well.

To verify that Survivin antagonists were not inducing non-specific toxicity, we tested their effects on GNPs, which express *survivin* (see Figure 1), and post-mitotic neurons, which do not. Treatment of GNPs with YM155 or S12 caused a dose dependent increase in the

percentage of dead cells (as measured by EthD1 staining). In contrast, survival of post-mitotic neurons was not affected by treatment with Survivin antagonists (Supp. Fig 3A,B). These data suggest that Survivin antagonists induce death of Survivin-expressing cells but are not toxic to normal cerebellar neurons.

## Survivin antagonists cooperate with radiation and SHH antagonists

Among the major drawbacks of current MB therapy are the devastating side effects of radiation(35, 36). Since recent studies have suggested that YM155 can enhance the efficacy of radiotherapy against NSCLC(37), we hypothesized that combining radiation and Survivin antagonists might be effective for MB as well. To test this, we performed thymidine incorporation assays on tumor cells treated with Survivin antagonists for 24hr followed by exposure to varying doses of radiation (Figure 5A). Treatment with sub-optimal doses of YM155 and S12 alone resulted in a small decrease in proliferation. The combination of Survivin antagonists and 0.25 grey (Gy) radiation markedly decreased tumor cell proliferation compared to radiation alone. The level of inhibition achieved with the combination treatment was equivalent to that achieved by doubling the radiation dose. These data suggest that Survivin antagonists can enhance the effects of radiation on MB cells, and raise the possibility that these agents might allow reduction in the doses of radiation used for therapy.

In addition to conventional therapy, targeted therapies have begun to be evaluated for treatment MB. For SHH-associated tumors, NVP-LDE225 a small molecule antagonist of SMO, has shown some efficacy in animal models as well as in patients(38, 39). To test whether Survivin antagonists increase the efficacy of SHH antagonists, we treated *Ptch* mutant tumor cells with various concentrations of LDE225 alone or in combination with 10μg/ml S12 (Figure 5B). LDE225 alone had an $IC_{50}$ of 5.5 nM (range: 3-8.5 nM) while the combination of LDE225 and S12 markedly decreased the $IC_{50}$ to 0.04 nM (range: 0.04-2 nM). Similarly, exposure to 20 nM YM155 decreased the $IC_{50}$ of LDE225 (from 13.5nM to 6.4nM). These data suggest that Survivin antagonists significantly enhance growth inhibition by LDE.

## Survivin antagonists inhibit growth of human SHH-driven MB cells

The studies described above focused on murine SHH-associated MB. To determine whether human SHH-driven MB cells also respond to Survivin antagonists, we used patient-derived xenografts (PDXs) from SHH-driven tumors. Real time PCR analysis revealed that PDX cells express high levels of *survivin* (Supplementary Figure 4). Treatment of PDX cells with Survivin antagonists *in vitro* led to significant decreases in $^{3}$H-thymidine incorporation (Figure 6A-C). Tumor cells were also treated with the SMO antagonist LDE225. Notably, the PDX line with a mutation upstream of SMO[7] (DMB-012) was responsive to LDE225 (Figure 6A, left panel and ref(40)) whereas the lines with mutations downstream of SMO(40),(41) (RCMB-018 and ICb-984MB) were resistant (Figure 6B,C). In contrast, all three lines responded robustly to YM155 and high dose S12. These data suggest that Survivin antagonists can inhibit the growth of human SHH-driven MBs and that they may be useful for treating tumors that are resistant to SMO antagonists.

### Survivin antagonists inhibit MB growth *in vivo*

Given the ability of Survivin antagonists to inhibit proliferation and promote apoptosis of tumor cells *in vitro*, we questioned whether inhibiting Survivin could prevent tumor growth *in vivo*. Unfortunately, published reports (60) as well as our own preliminary studies suggested that these antagonists do not accumulate in the brain or intracranial tumors. Therefore, *Ptch* mutant tumors were implanted into the flanks of Nu/Nu mice and treated with YM155 or vehicle. Intratumoral injections of YM155 significantly decreased tumor growth compared to treatment with vehicle (Fig7A). Tumors harvested after 6 weeks of treatment were much smaller than those in the vehicle treated mice (Fig7B, C). We also tested whether systemic treatment with YM155 could inhibit tumor growth *in vivo*; since YM155 has a short half-life, we used osmotic pumps for delivery(20, 32, 42). Nu/Nu mice bearing *Ptch* mutant flank tumors were implanted with micro-osmotic pumps to continuously infuse YM155 or vehicle (saline) for 3 weeks. Tumors in mice with YM155-containing pumps grew significantly less than those in mice with vehicle pumps (Fig 7D-F). These data suggest that Survivin antagonists can potently inhibit MB growth *in vivo*.

## Discussion

Previous studies have shown that Survivin is expressed in human MB, and that its expression is correlated with poor outcome. To study the role of Survivin in MB growth and survival, we used a mouse model of MB driven by activation of the SHH pathway. Our studies demonstrate that Survivin is highly expressed in SHH-driven MB and that genetic and pharmacologic inhibition of Survivin impedes growth of MB cells *in vitro* and *in vivo*.

Our initial studies demonstrated that Survivin is expressed at high levels in *Ptch* mutant MB cells and not in normal adult cerebellum, suggesting that it might represent a cancer-selective target. To test the functional importance of Survivin in MB cells, we used Cre viruses to delete Survivin from $Survivin^{f/flx};Ptch^{+/-}$ tumor cells. Loss of Survivin resulted in markedly decreased proliferation and arrest in the G2/M phases of the cell cycle. These results are consistent with the function of Survivin as a member of the chromosomal passenger complex, which is critical for alignment of chromosomes during metaphase and for successful cell cleavage(43, 44). In addition, previous studies have shown that loss of Survivin leads to aberrant mitosis, centrosome amplification and failed cytokinesis in a number of cancers, including glioma and cervical cancer(45, 46). Thus, in MB as in other tumors, Survivin seems to be critical for normal proliferation and cell cycle progression.

To evaluate Survivin's utility as a therapeutic target, we used small molecule antagonists. Consistent with our genetic studies, Survivin antagonists significantly decreased proliferation and altered cell cycle progression. Interestingly, while S12 treatment caused accumulation of cells in G2/M phase (similar to *survivin* deletion), YM155 caused cells to accumulate in S-phase. This observation is consistent with previous studies that demonstrated YM155 treatment can cause a loss of new DNA synthesis and concomitant stall in S phase(47). This discrepancy between S12 and YM155 could be due to differences in the kinetics or degree of Survivin inhibition induced by these drugs. Alternatively, it could result from differences in the mechanisms by which the drugs act: whereas S12 binds directly to Survivin protein(33), YM155 inhibits *survivin* expression by disrupting ILF3/

NF110 complexes(48, 49) or Sp1 binding at the *survivin* promoter(50). Thus, in addition to potently decreasing *survivin* expression (see Ref #32 and sup fig 1), YM155 may also alter expression of other genes that regulate cell cycle progression (e.g. cyclin D1, p27) (51, 52), and thereby cause arrest at earlier stages of the cycle. Nonetheless, the fact that genetic deletion and several small molecule inhibitors all interfere with cell cycle progression in *Ptch* mutant tumor cells strongly supports the notion that Survivin is required for this process in SHH-driven MB.

In addition to their effects on the cell cycle, Survivin antagonists also increased the percentage of MB cells undergoing apoptosis. It remains unclear whether loss of Survivin function causes apoptosis directly or as a consequence of cell cycle arrest. Analysis of Annexin+ cells after treatment with Survivin antagonists showed that apoptosis is not detectable until 36hr, whereas cell cycle inhibition is observed by 12-24hr. These results are consistent with the notion that apoptosis occurs secondary to cell cycle arrest. The fact that Survivin antagonists not only inhibit proliferation but also cause death of tumor cells may make them potent therapeutic agents for MB.

Previous studies of glioma and other cancers have suggested that targeting Survivin can enhance the effects of radiation and chemotherapy(20, 37, 53-58). In agreement with these data, we found that Survivin antagonists significantly enhanced the sensitivity of MB cells to radiation. Combining low dose radiation (0.25 grey) with S12 or YM155 was as effective at inhibiting tumor growth as doubling the dose of radiation. These data suggest that addition of Survivin antagonists may allow children to be treated with lower doses of radiation without decreasing therapeutic benefits. The approach could markedly reduce treatment-related side effects and improve the long term quality of life of MB patients.

A major advance in treatment of SHH driven MB has been the development of SMO antagonists. However, one drawback of these agents is the swift acquisition of resistance to the drug and tumor recurrence. Our studies show that combining LDE225 and Survivin antagonists significantly enhanced inhibition of proliferation compared to either drug alone. Although in these studies we focused on co-treatment, it would be interesting to look at the ability of Survivin antagonists to overcome LDE225 resistance. In this context, it is notable that in non-small cell lung cancer, downregulation of Survivin by either siRNA or treatment with YM155 reverses Erlotinib resistance(59). It is also important to note that LDE225 and other SHH antagonists work at the level of SMO, and are thus ineffective for patients with mutations downstream in the SHH pathway(7). We show that human PDX cells with such downstream mutations are still sensitive to inhibition by Survivin antagonists. In addition, our preliminary studies suggest that that YM155 can inhibit growth of non-SHH-associated MB cells (data not shown). These studies raise the possibility that Survivin may be a therapeutic target for a broad spectrum of MB patients.

Lastly, we have shown that YM155 can inhibit tumor growth *in vivo*, either by direct intratumoral injection or systemic administration. These data strongly suggest that targeting Survivin could be an effective approach for treating MB. Unfortunately, pharmacokinetic studies by our lab and others ((60) and data not shown) suggest that the Survivin antagonists we have tested do not show significant accumulation in the brain or in intracranial tumors.

Thus, chemical modification of these agents, or alternative modes of delivery (e.g. convection-enhanced(61, 62) or intrathecal delivery(63, 64)) may be necessary to make these agents useful for treatment of MB and other brain tumors.

## Materials and Methods

### Mice

*Survivin*$^{fl/fl}$ (31), *Ptc*$^{fl/fl}$ (65), *Math1-Cre-ER*(66)' *Ptch* +/-(67), and *Math1CreER;ptc*$^{fl/fl}$ (30) (MERP) mice have been described previously. P4 MERP pups were gavaged with 0.8g/40μl of tamoxifen (T-5648, Sigma, St. Louis, MO) in corn oil to generate tumors. Tumors from *Ptch*$^{+/-}$ and MERP mice were used for experiments. To allow for deletion of *survivin* in tumor cells, *Survivin*$^{fl/fl}$ mice were crossed with *Ptch*$^{+/-}$ mice to generate the *Survivin* $^{fl/fl}$*; Ptch*$^{+/-}$ (SP) line. CD-1 Nu/Nu mice were from Charles River Laboratories (Wilmington, MA). P7 wild type C57BL/6 pups were obtained from the SBMRI Animal Facility. All mice were maintained in the Animal Facility, and experiments were performed in accordance with national guidelines and regulations, and with the approval of the SBMRI Institutional Animal Care and Use Committee.

### Cell isolation and in vitro culture

GNPs were isolated from P7 cerebellum and tumors from adult cerebellum as previously described(30, 68). Briefly, tissue was digested in a solution containing 10 U/ml papain (Worthington Biochemical Corporation, Lakewood, NJ) and 250 U/ml DNase (Sigma), and triturated to obtain a single-cell suspension. Cells were spun through a 35-65% Percoll gradient (GE Healthcare Uppsala, Sweden) to purify GNPs and tumor cells. Cells were cultured in NB/NS-21 media (Neurobasal media, 1 mM sodium pyruvate, 2 mM L-glut, penicillin/streptomycin and NS-21 supplement, plus 1% FBS (Invitrogen Grand Island, NY)) on growth factor-reduced (GFR) matrigel-coated plates (1:50 in NB/NS-21, BD Biosciences, San Diego, CA).

### Real-Time PCR

For analysis of *survivin* expression, mRNA was isolated from cells and tissues using an RNAeasy Plus Mini kit (QIAGEN Inc, Valencia, CA). One-step qRT-PCR reactions were performed in triplicate using QuantiTech RT mix (QIAGEN) on the Bio-Rad C1000 Thermocycler and CFX96 system (Bio-Rad Laboratories, Hercules, CA). Duplicate reactions were prepared without reverse transcriptase to confirm the absence of genomic DNA contamination. Relative gene expression was calculated using the ΔΔCT method and normalized to Actin. 95% confidence intervals for each sample were calculated using the sum of the squares method. To evaluate the efficiency of *survivin* deletion by Cre infection, SP tumor cells were isolated, infected with Cre-IRES-GFP or GFP retroviruses for 48hrs, sorted for GFP expression, and analyzed as outlined above.

### Immunohistochemistry

For staining of paraffin-embedded tissue, animals were perfused with PBS followed by 4% paraformaldehyde (PFA, Affymetrix, cat# 19943). Cerebella were removed, fixed in 4% PFA overnight and delivered to the SBMRI Histology Shared Resource for embedding,

antigen retrieval, and staining. Sections were stained either with anti-Survivin antibodies (Cell Signaling Technology Cat#2808S, Danvers, MA) alone or anti-Survivin antibodies pre-incubated with Survivin blocking peptides (Cell Signaling Technology Cat #1037).

### Cell Lysis and Western-blotting

To evaluate Survivin expression, tumor cells, GNPs and adult cerebellum were lysed in RIPA buffer (Millipore, Billerica, MA). Protein was quantitated using the Bio-Rad protein assay. Equal amounts of protein were separated by SDS-PAGE, blocked with 5% BSA (Sigma) in Tris-buffered saline with 0.1% Tween-20 (TBST), and stained with anti-Survivin or GAPDH antibodies overnight (1:1000, Cell Signaling Technology Cat# 2808S, 5174) followed by anti-rabbit HRP-conjugated secondary antibody (1:2000 Cell Signaling technology, Cat# 7074S). Proteins were visualized by incubating with Pierce ECL plus (Thermo Fisher Scientific, Rockford, IL). To evaluate Survivin expression after YM155 treatment, tumor cells were plated on 6-well plates (6-8M cells/well) and treated with YM155 or DMSO (Fisher Scientific Inc. San Diego, CA) at 1 μM for 24hrs and processed as described above.

### Analysis of proliferation, cell cycle and apoptosis

To analyze the effects of Survivin loss, cells were isolated from SP tumors and infected with Cre-IRES-GFP or GFP retroviruses (MSCV, 1:5 in media). To assess the effects of pharmacological inhibition of Survivin, *Ptch* mutant tumor cells were treated with YM155 (Selleck Chemicals), S12(33), LLP3(34), or DMSO (Fisher Scientific) at the indicated concentrations.

**Ki67 staining—**Cells were plated on GFR matrigel-coated chamber slides at 0.2M cells/well and treated with DMSO or antagonists for 24hrs. Cells were fixed in 4% PFA, permeabilized with 0.1% Triton X-100 in PBS (Aqua Solutions Deer Park, TX) and blocked with 10% goat serum (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) before staining with anti-Ki67 (BD Biosciences Cat# 556003) and 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). Six representative images from each treatment were collected using the Zeiss LSM-700 confocal and Ki67+ percentages calculated using ImageJ software (NIH).

**Thymidine incorporation—**Cells were plated ($2 \times 10^5$ cells/well) in GFR-matrigel coated 96 well plates and treated with either virus or antagonists for 48hrs in triplicate wells before being pulsed with methyl-[$^3$H]thymidine (GE Healthcare, Piscataway, NJ, USA). After 12-16 hr, cells were harvested using a Mach III manual harvester 96 (Tomtec, Hamden, CT, USA), and incorporated radioactivity was quantitated using a Wallac MicroB microplate scintillation counter (Perkin Elmer, Waltham, MA, USA).

**Cell cycle analysis—**Cells were plated in GFR-matrigel coated 48 well plates at 0.4M cells/well, infected with virus or treated with antagonists, and collected at various time points. Cells were fixed and stained using the FITC BrdU Flow Kit (BD Biosciences) and 7-Aminoactinomycin (7-AAD) according to the manufacturer's instructions. Analysis was performed using a FACSCanto II flow cytometer (BD Biosciences) and FlowJo v.7.6.4 software (Tree Star, Inc., Ashland, OR).

**Apoptosis—**Tumor cells were plated on 24-well plates at 1M cells/well and treated with Survivin antagonists or infected with viruses for 36 hr. Cells were collected by incubating with papain solution and resuspended in 100 ul of Annexin-binding buffer containing 5μL of AnnexinV conjugate (Annexin-FITC or Annexin-567, both Invitrogen) and 1μl Propidium iodide (PI, 1.0mg/ml stock, Invitrogen). Cells were analyzed using a FACSCanto II and FlowJo v.7.6.4 software.

**Live/Dead Assay—**To address toxicity of YM155, GNPs were isolated from WT P7 pups and plated at $0.2 \times 10^6$ cells/well in 2 96 well plates coated with GFR-matrigel for each experiment. One plate was maintained in proliferation media consisting of NB/NS-21 and Sonic hedgehog (SHH)-containing supernatant (1:5 in media) and treated with either DMSO or various doses of YM155 and S12 with each condition in triplicate wells. After 48hrs, cell viability was analyzed using the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen). Briefly, cells were stained with 4μM EthD1 for 40 min and fluorescence emission (645 nM) was measured using a TECAN infiniteM200 Microplate reader (Morrisville, NC). The second plate was maintained in differentiation media (NB/NS-21 containing 25mM glucose and 25mM potassium chloride) for 5 days to produce post-mitotic neurons. Cells were then treated with DMSO or corresponding doses of YM155 and cell viability was evaluated after 48hrs.

## Radiation and LDE225 treatment

To measure effects of inhibitors in combination with radiation, tumor cells were plated in 96-well plates at a density of $0.2 \times 10^6$ cells per well and cultured in the presence of DMSO, 50 nM YM155, or 10 ug/ml S12. After 24hrs, cells were subjected to 0, 0.25, or 0.5 Gy radiation using a Gammacell 40 Exactor (Low-dose cesium 137 irradiator, Best Theratronics Ltd., Ottawa, Ontario, Canada). Cells were cultured for an additional 24 hr, and [methyl-$^3$H]thymidine assays were performed as described above.

To measure effects of inhibitors in combination with the SHH antagonist NVP-LDE225 (Selleck Chemicals, S2151), tumor cells were plated in 96 well plates at $0.2 \times 10^6$ cells/well and cultured with increasing doses of LDE225 or a single dose of Survivin antagonist (10μg/ml S12, 20nM YM255) alone or in combination with LDE225 as indicated. Cells were cultured for 48hrs and [methyl-$^3$H]thymidine assays were performed as described above.

## Human tumor isolation, propagation, and classification

Human MB tissue for patient-derived xenografts was obtained from surgical resection of tumors at Duke University Medical Center (Durham, NC), Rady Children's Hospital (San Diego, CA) or Texas Children's Cancer Center (Houston, TX). All procedures using human tissue were approved by the Institutional Review Boards of the respective institutions. Upon retrieval, the tissue was mechanically dissociated into a single-cell suspension, then immediately injected into the cerebella of NSG mice. When mice showed signs of MB, tumors were again dissociated into single-cell suspensions and re-transplanted back into the cerebella of naïve hosts to establish a propagated line for each patient-derived xenograft. Molecular classification of human tumors was previously described(7, 40, 41)

### Flank tumor implantation and in vivo antagonist treatment

Cells isolated from tumors were re-suspended 1:1 in NB/NS-21 media and GFR-matrigel. 100μl of cell suspension (6-7 × $10^6$ cells) was injected subcutaneously into the flanks of 5-8 week old CD-1 Nu/Nu mice. Tumor growth was monitored using calipers and tumor volume calculated using the formula 0.52*length*$width^2$. Treatment was initiated when tumors reached ∼100 $mm^3$. For intratumoral injections, tumors were injected twice a week with YM155 (20 μM final concentration) or vehicle (20% DMSO in saline). For systemic treatments, mice were treated with 10 mg/kg/day YM155 or saline by subcutaneous micro-osmotic pump (Alzet, model D2004). Experimental treatments were continued until control tumors reached maximum size of 2000$mm^3$, at which point tumors were collected for analysis.

### Statistics

Unless otherwise indicated, statistics were calculated by ANOVA with post hoc student's t-test. p values of less than 0.05 were considered significant and marked with asterisks where appropriate.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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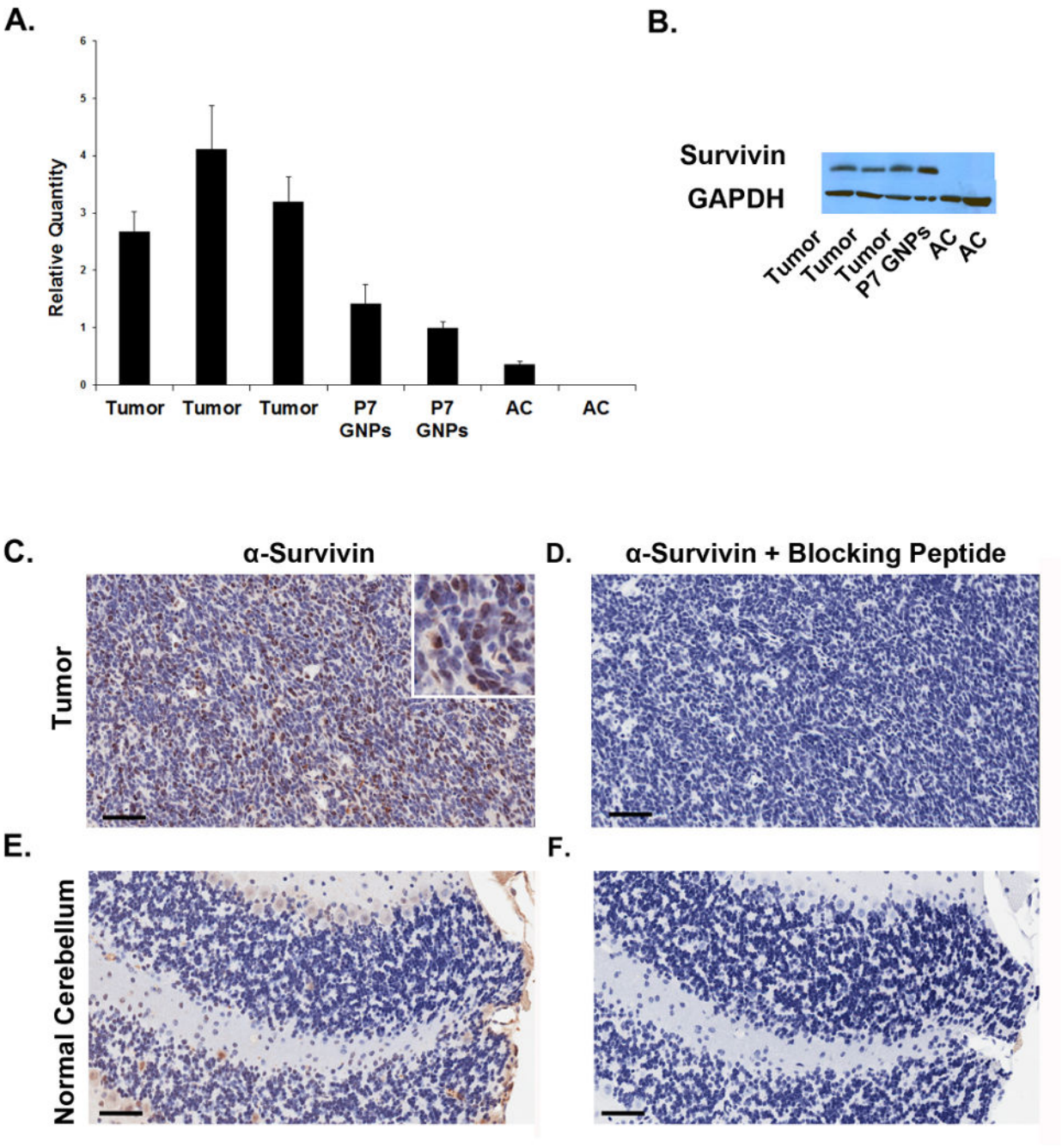



**Figure 1. Survivin is expressed in *Ptch* mutant tumors**

RNA and protein from *Ptch* mutant MB cells, P7 GNPs and adult cerebellum were analyzed for Survivin expression using real time PCR (A) and by western blotting (B). Survivin is highly expressed in tumors and GNPs, but not in adult cerebellum. Error bars in (A) represent 95% confidence interval calculated using sum of the squares method ($p<0.02$ by ANOVA and post hoc student's t-test). (C-F) Tissue sections from *Ptch* mutant tumor and normal adult cerebellum were stained with anti-Survivin antibodies alone (C,E) or with anti-Survivin antibodies that were pre-incubated for 30 min with Survivin blocking peptide

(D,F). Survivin is highly expressed in tumor cells with minimal expression in adult cerebellum. Inset in (C) is 4x magnification of positive staining. Scale bars represent 50 μM. Data are representative of 3 experiments.

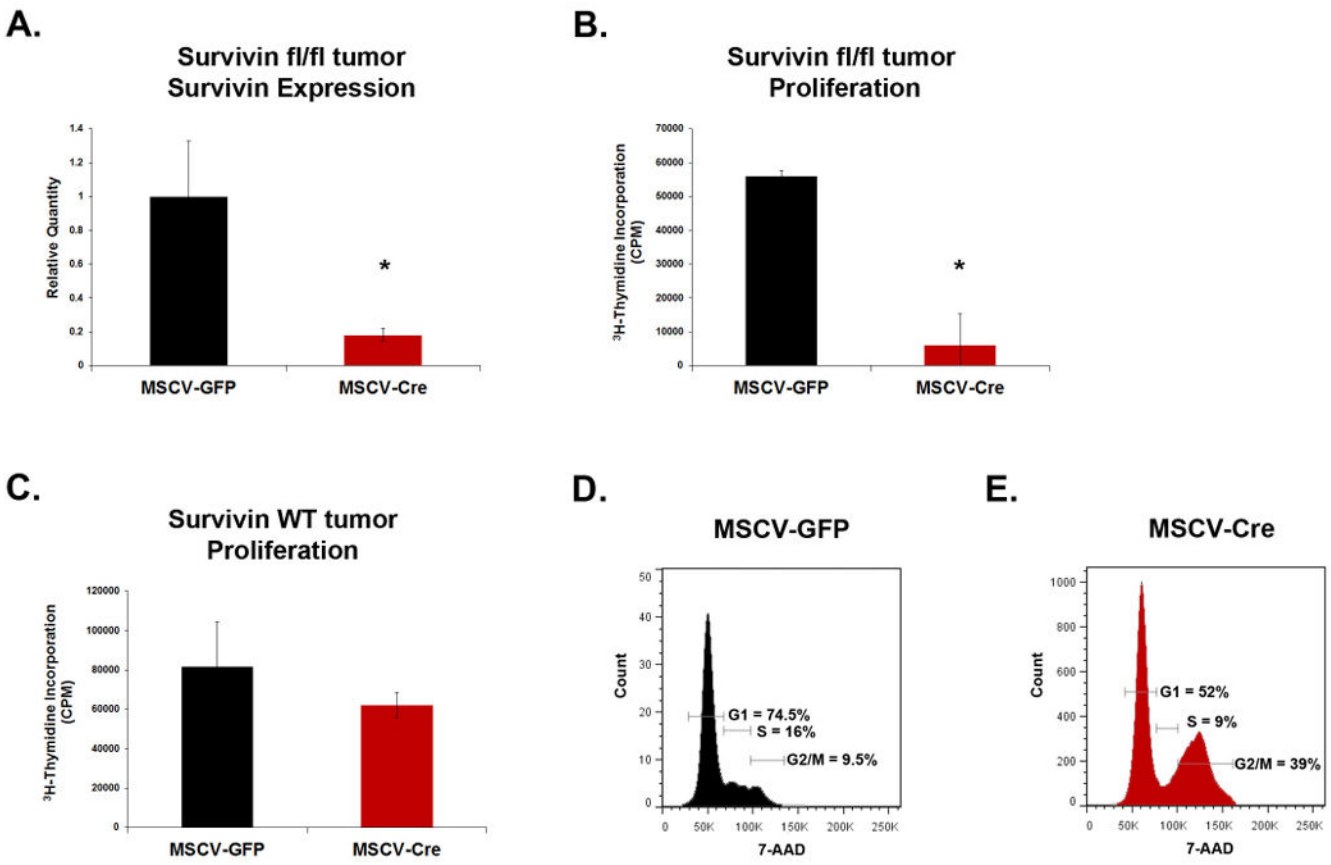



**Figure 2. Loss of Survivin causes decreased proliferation and cell cycle arrest**

(A-B) Cells were isolated from *Survivin*$^{fl/fl}$;*Ptch*$^{+/-}$ tumors and infected with Cre- or GFP retroviruses for 48 hr. (A) GFP+ cells were isolated by flow cytometry and *survivin* mRNA expression analyzed by RT-qPCR (n=2). Cre causes loss of *survivin* expression ($p<0.02$). (B) Cells were pulsed with $^{3}$H-thymidine for 12 hr, harvested, and analyzed for incorporation. Loss of *survivin* leads to decreased tumor cell proliferation ($p<0.001$). Data are representative of 5 experiments. (C) Cells were isolated from *Ptch*$^{+/-}$ tumor (wild type for survivin), infected with Cre- or GFP viruses for 48 hr, and collected after 12 hr pulse with $^{3}$H-thymidine to measure incorporation. Infection with Cre virus alone does not significantly impair proliferation ($p>0.1$). Data in (A-C) represent mean +/- standard deviation (SD) and are representative of 4 experiments. (D, E) Cells from *Survivin*$^{fl/fl}$;*Ptch*$^{+/-}$ tumors were infected with virus as described above (D. GFP, E. Cre virus) and stained with 7-AAD for cell cycle analysis by flow cytometry. *survivin* deletion causes accumulation of cells in G2/M. Data are representative of 4 experiments and cell cycle percentages based on live cell gates (excluded subG1). p values calculated using student's t-test.

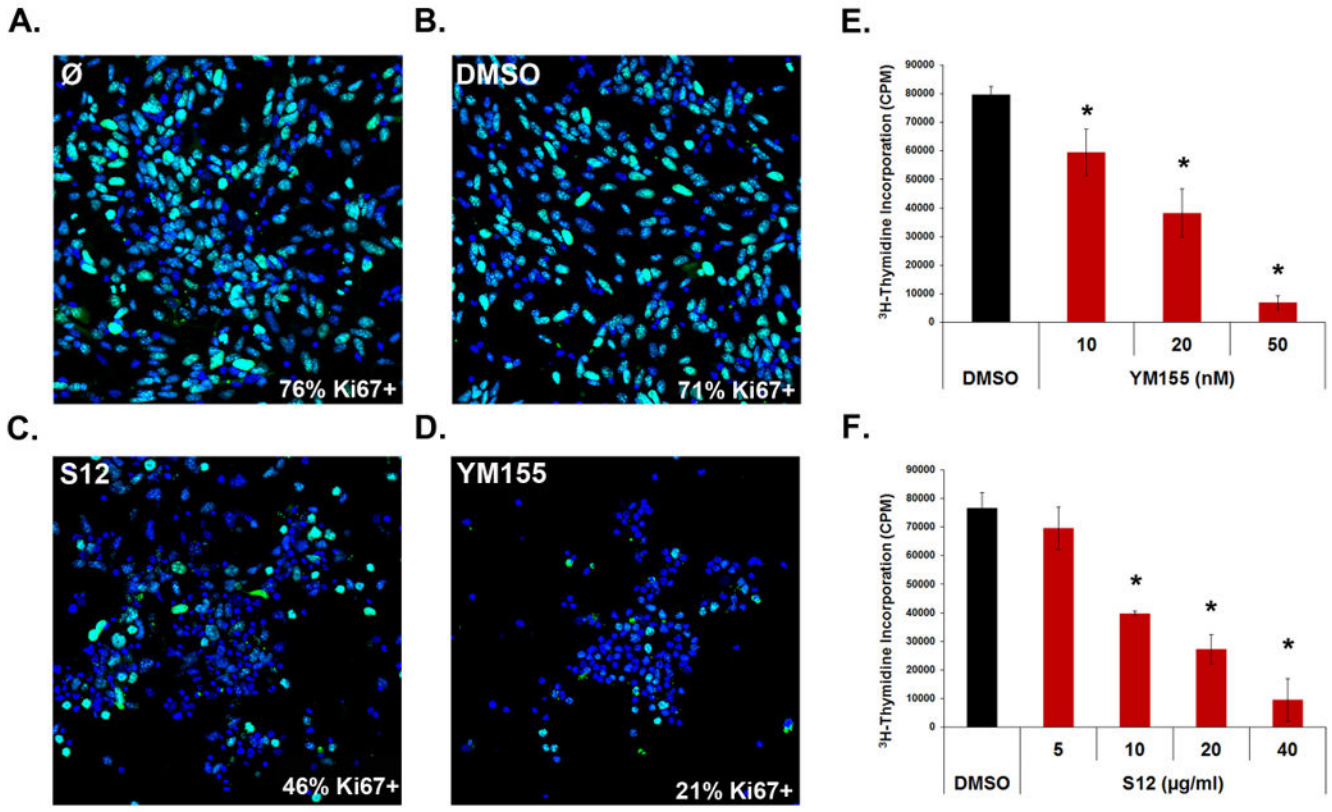



**Figure 3. Survivin antagonists inhibit proliferation**

(A-D) Tumor cells were plated on chamber slides and treated for 24 hr with control media (A), 0.1% DMSO (B), 10 µg/ml S12 (C), or 50 nM YM155 (D). Cells were stained with anti-Ki67 antibodies (green) to mark proliferating cells and DAPI (blue) to label cell nuclei. Very few cells were Ki67+ after treatment with Survivin antagonists compared to controls ($p<0.0001$ for YM155 and S12). Data are representative of 3 experiments. (E-F) Ptch mutant tumor cells were treated with multiple doses of YM155 (E) or S12 (F) for 48 hr and pulsed with 3H-thymidine for 12 hr to measure proliferation. Treatment with either antagonist decreased proliferation in a dose-dependent manner ($p< 0.02$ for all YM155 and S12 doses except 5µg/ml S12, which was not significant (NS)). Ki67+ percentages in (A) were averaged from 6 images for each treatment. Data in (E-F) represent mean +/- SD and are representative of 6 experiments. Stats were calculated by ANOVA and post hoc student's t-tests.

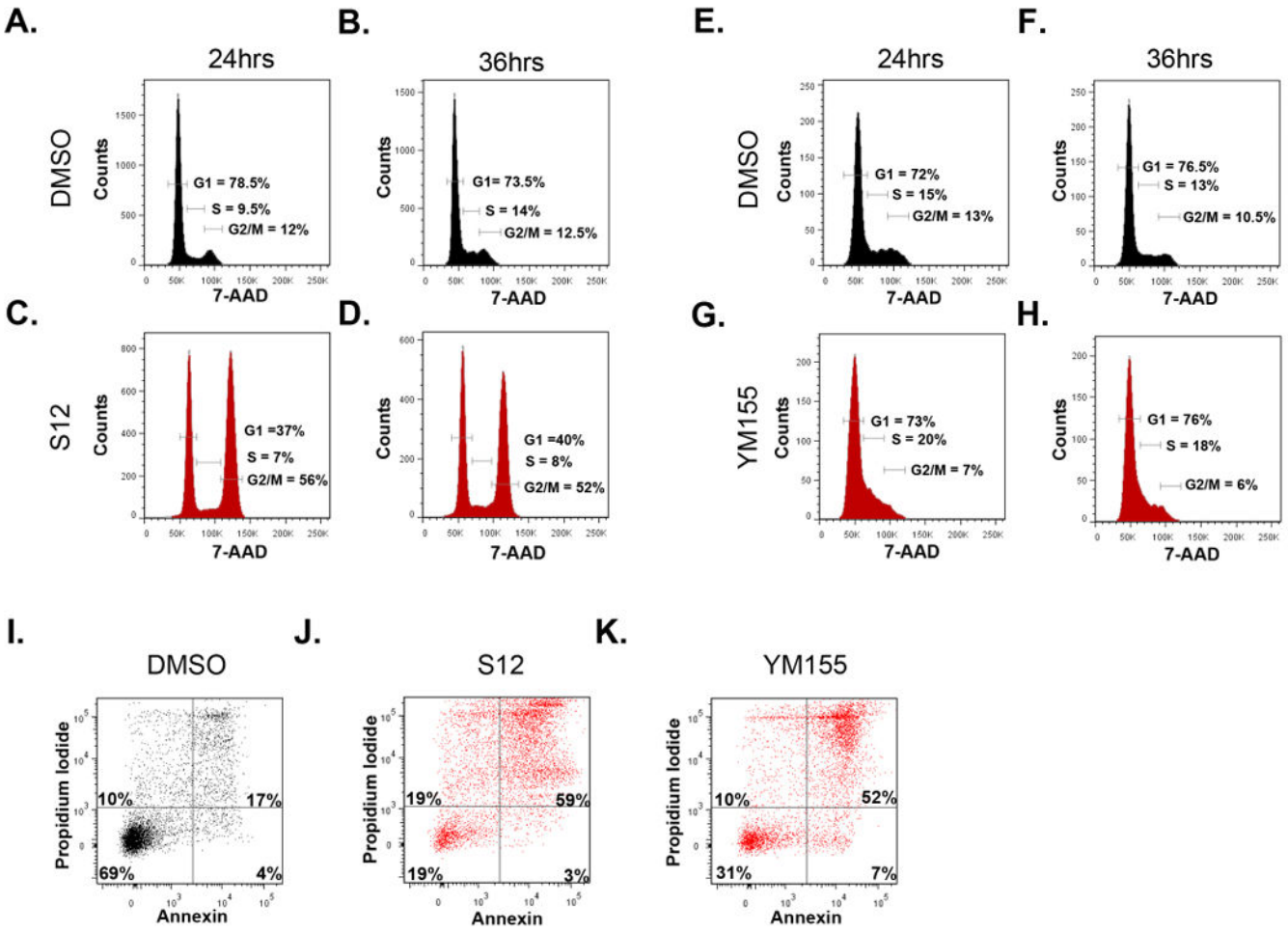
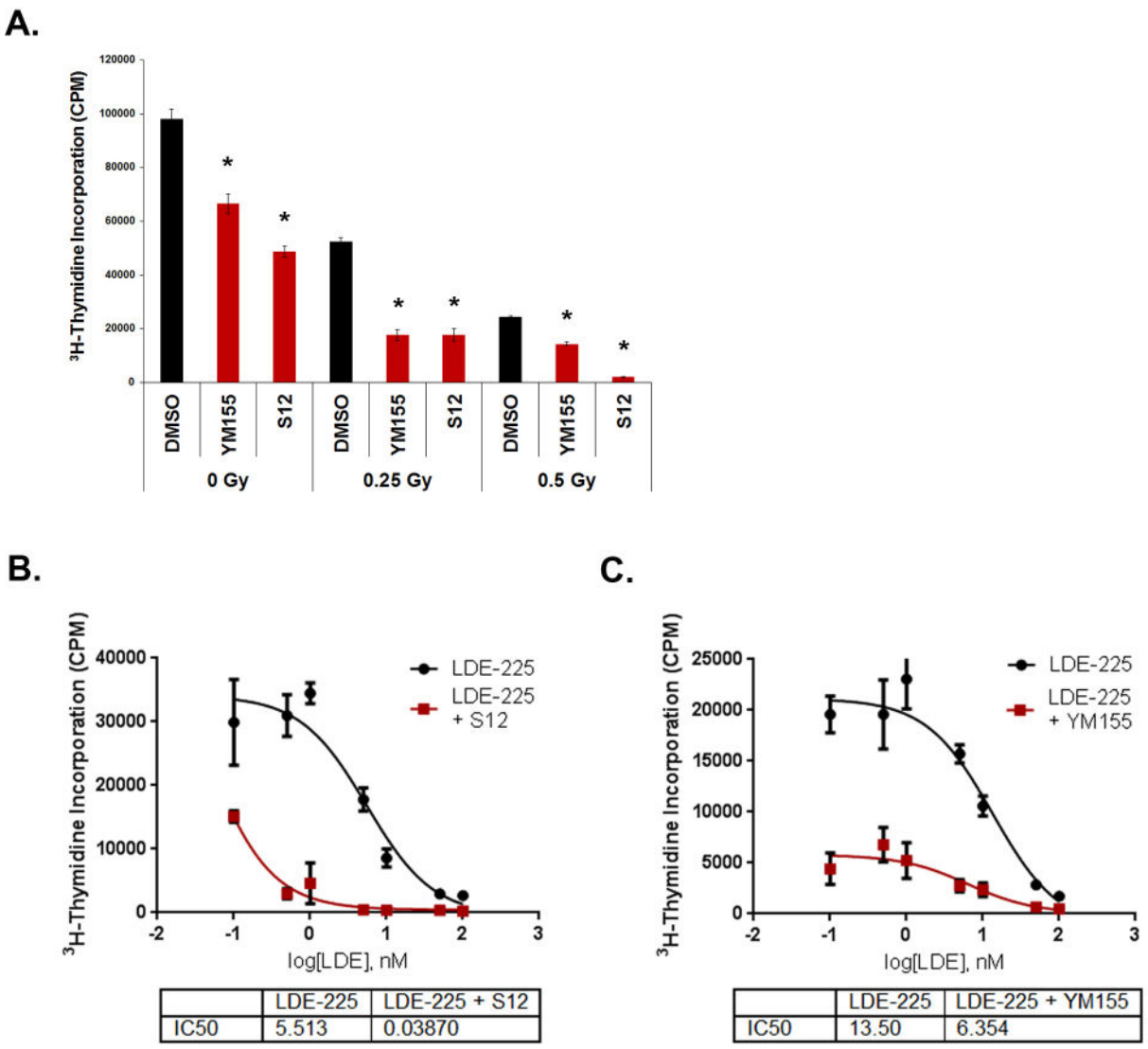



**Figure 4. Survivin antagonists alter cell cycle progression and promote apoptosis**

(A-H) *Ptch* mutant tumor cells were treated with either DMSO (A,B,E,F), 20 μg/ml S12 (C,D), or 100 nM YM155 (G,H) and stained with 7-AAD for cell cycle analysis after 24 hr (A,C,E,G) or 36 hr (B,D,F,H). YM155 decreased the percentage of cells in G2/M, while S12 treatment caused an accumulation of cells in G2/M. Data represent 4 (A-D) and 6 (E-H) experiments and percentages based on live cell gates (excluded subG1). (I-K) Tumor cells were treated with DMSO (I), 20 μg/ml (J), or 100nM YM155 (K) for 36 hr, then collected and stained with Propidium Iodide (PI) and Annexin-V for FACS analysis. The percentage of apoptotic cells was significantly higher after antagonist treatment compared to control. Data represent 6 independent experiments.




| | LDE-225 | LDE-225 + S12 |
|---|---|---|
| IC50 | 5.513 | 0.03870 |

| | LDE-225 | LDE-225 + YM155 |
|---|---|---|
| IC50 | 13.50 | 6.354 |

**Figure 5. Survivin antagonists cooperate with radiation and LDE225 SHH antagonists**

A) Tumor cells were treated with either 0.1% DMSO, 50 nM YM155, or 10 μg/ml S12 for 24 hr, and then irradiated with 0, 0.25, or 0.5 gray (Gy). After 24 hr, cells were pulsed with $^3$H-thymidine and assayed for incorporation. Treatment with antagonists enhanced sensitivity of tumor cells to radiation ($p<0.02$ for all treatments, calculated by 2 way ANOVA to identify radiation dose by treatment interaction, split by radiation dose, with post hoc student's t-tests) Data are representative of 5 experiments. (B,C) Tumor cells were plated in 96 well plates and treated with LDE225 alone (at the indicated concentrations) or in combination with 20 nM YM155 (B) or 10 μg/ml S12 (C). Cells were pulsed with $^3$H-thymidine after 48 hr and harvested to assay levels of incorporation. Combination treatment of LDE with either S12 or YM155 significantly lowered the IC50 compared to LDE alone ($p< 0.01$ by student's t-test). Data are representative of 4 (B) and 3 (C) experiments. All data represent mean +/- SD.

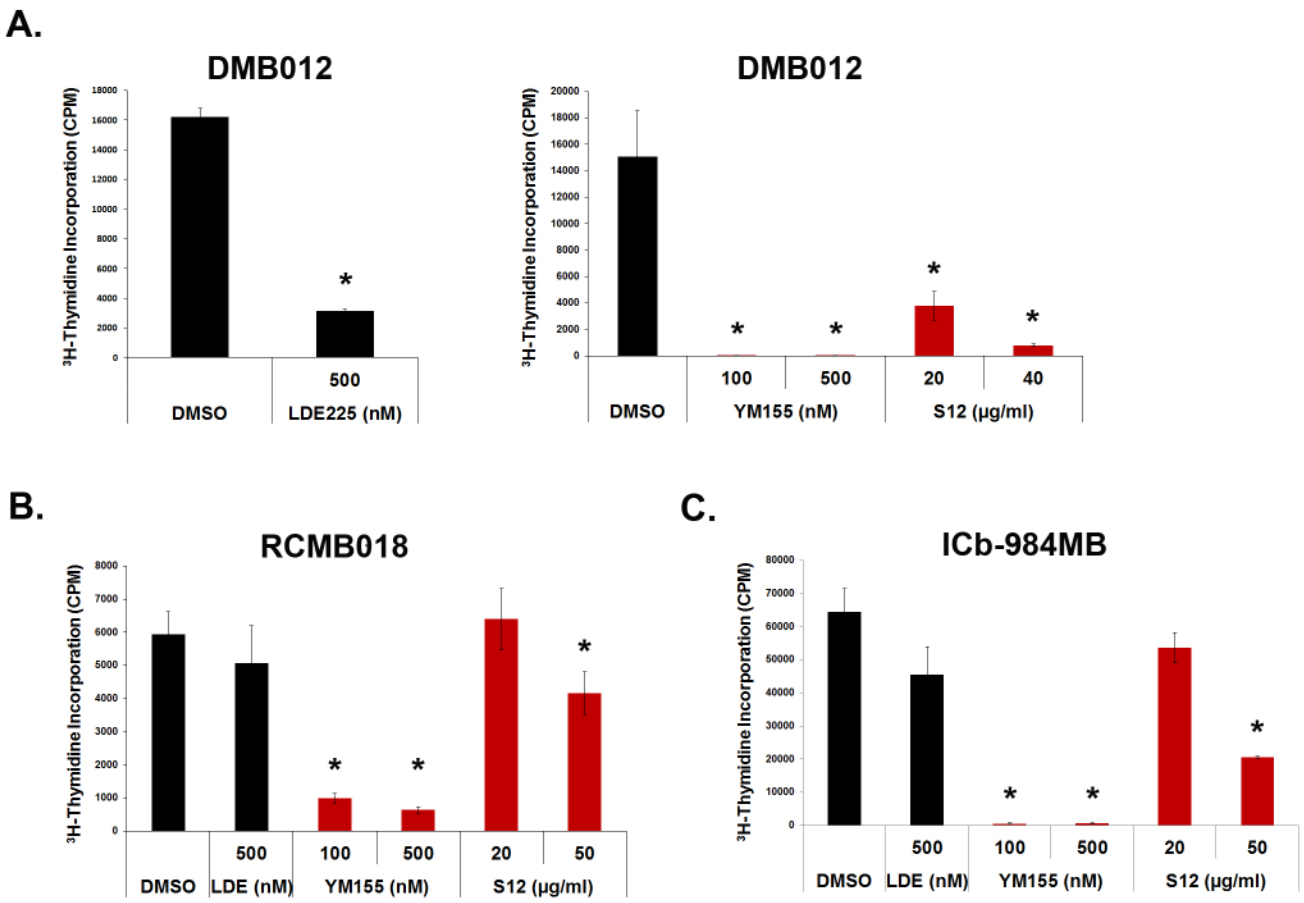



**Figure 6. Survivin antagonists inhibit proliferation of human SHH-driven MB cells**

Cells isolated from LDE225-sensitive xenograft DMB012 (A) and LDE-insensitive xenografts RCMB018 (B) and ICb-984MB (C) were treated for 48 hr with DMSO, LDE225, YM155 or S12, and analyzed for thymidine incorporation following a 12-16 hr pulse. All tumor cells were sensitive to YM155 inhibition and high dose S12 treatment (DMB012 $p<0.01$ for all doses including LDE225, RCM018 and ICb-984MB $p<0.03$ for YM155 and 10µg/ml S12 while LDE and 20µg/ml S12 were not significant with $p>0.08$). All stats were calculated by ANOVA and post hoc student's t-test. Data represent mean +/- SD and are representative of 3 experiments.

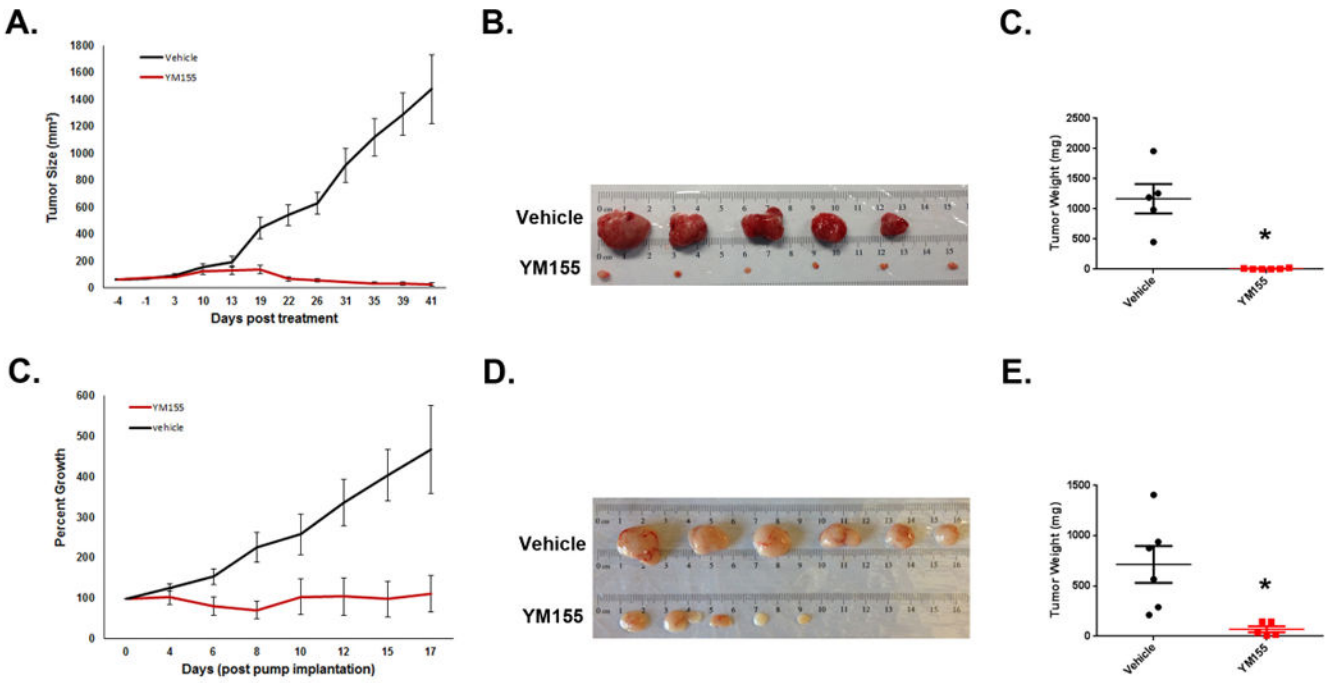



**Figure 7. YM155 inhibits growth of Ptch mutant tumor cells in vivo**

(A-F) Tumor cells were suspended in GFR matrigel (1:1 with media) and implanted in the flanks of Nude mice. When tumors reached ~100mm$^3$, mice were split into two cohorts and treatment was started. For (A-C), tumors were treated with vehicle (20% DMSO in saline) or YM155 (20 μM) via intratumoral injection twice a week (vehicle n=5, YM155 n=6). For (D-F), mice were treated with vehicle (saline) or YM155 (10 mg/kg/day) via micro-osmotic pump (vehicle n=6, YM155 n=5). Caliper measurements were made twice a week to monitor tumor growth (A,D) and resulting tumors were collected, photographed (B,E), and weighed (C,F). Experiments were repeated 2 (A-C) and 3 times (D-F) respectively. Both intra-tumoral and systemic treatment with YM155 decreased tumor size over time compared to vehicle control. ($p<0.02$ for IT and pump YM155 tumor weight by ANOVA with post hoc student's t-test). Error bars represent SEM.